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WITNESS my hand this
Thirtieth day of June 2004

J. Billingsley

JULIE BILLINGSLEY
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PROVISIONAL SPECIFICATION

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Invention Title: **Analogues of heteromeric proteins**

The invention is described in the following statement:

ANALOGUES OF HETEROMERIC PROTEINS

Field of the Invention

This invention relates to analogues of heteromeric proteins, including in a preferred form analogues of the relaxin superfamily, including analogues which are agonists or antagonists at the relaxin superfamily receptor(s).

The relaxin superfamily currently comprises 10 members with a relatively high degree of sequence homology. These family members include insulin, insulin-like grown factors I and II, relaxin 1, 2 and 3, INSL3, 4, 5 and 6. The relaxin superfamily members have a wide range of biological activities which are well described in the art.

The critical action of insulin has been described for decades. Insulin has a key role in cellular metabolism. Many subjects with diabetes require daily administration of insulin.

The actions of relaxin include an ability to inhibit myometrial contractions, to stimulate remodelling of connective tissue and to induce softening of the tissues of the birth canal. Additionally, relaxin increases growth and differentiation of the mammary gland and nipple and induces the breakdown of collagen, one of the main components of connective tissue. Relaxin decreases collagen synthesis and increases the release of collagenases (Unemori et al (1990) *J. Biol. Chem.* 265, 10682-10685). These findings were recently confirmed by the establishment of the relaxin gene-knockout mouse (Zhao et al (1999) *Endocrinology* 140, 445-453), which exhibited a number of phenotypic properties associated with pregnancy. Female mice lacking a functionally active relaxin gene failed to relax and elongate the interpubic ligament of the pubic symphysis and could not suckle their pups, which in turn, died within 24 hours unless cross-fostered to relaxin wildtype or relaxin heterozygous foster mothers.

Evidence has accumulated to suggest that relaxin is more than a hormone of pregnancy and acts on cells and tissues other than those of the female reproductive system. Relaxin causes a widening of blood vessels (vasodilatation) in the kidney, mesocaecum, lung and

peripheral vasculature, which leads to increased blood flow or perfusion rates in these tissues (Bani et al (1997) *Gen. Pharmacol.* 28, 13-22). It also stimulates an increase in heart rate and coronary blood flow, and increases both glomerular filtration rate and renal plasma flow (Bani et al (1997) *Gen. Pharmacol.* 28, 13-22). The brain is another target tissue for relaxin where the peptide has been shown to bind to receptors (Osheroff et al (1991) *Proc. Nal. Acad. Sci. U.S.A.* 88, 6413-6417; Tan et al (1999) *Br. J. Pharmacol* 127, 91-98) in the circumventricular organs to affect blood pressure and drinking (Parry et al (1990) *J Neuroendocrinol.* 2, 53-58; Summerlee et al (1998) *Endocrinology* 139, 2322-2328; Sinnahay et al (1999) *Endocrinology* 140, 5082-5086).

Important clinical uses arise for relaxin in various diseases responding to fibrotic breakdown, connective tissue remodelling, vasodilation, such as coronary artery disease, peripheral vascular disease, kidney disease associated with arteriosclerosis or other narrowing of kidney capillaries, or other capillaries narrowing in the body, such as in the eyes or in the peripheral digits, the mesocaecum, lung and peripheral vasculature, and neurological modification.

The INSL peptides, such as INSL3, may be involved in various physiological actions, including descent of the gonads.

The relaxin superfamily members are all heteromeric proteins, comprising an A and B chain, which are disulphide bonded or linked.

There is a need for agonists and antagonists at the relaxin superfamily members. Agonists and antagonists of the various relaxin superfamily members provide the opportunity for a wide range of pharmacological actions and therapeutic treatments/treatment outcomes.

Summary of the Invention

In its broadest form, the present invention provides a method of producing an analogue of a heteromeric protein with a binding activity at a target site, the heteromeric protein having a total subunit number of n, the method including:

- synthesizing one or more target-site binding subunits of the heteromeric protein, wherein the maximum number of active subunits identified is n-1; and
- identifying at least one set of a first and a second amino acid on respective, opposing strands of a turn and/or loop moiety in the or each active subunits of the protein, with an alpha-alpha or beta-beta carbon separation distance of less than six angstroms; and
- introducing a chemical cross-link between the first and second amino acids of the at least one set of first and second amino acids;

wherein the chemical cross-link stabilises a three dimensional structure of at least one subunit.

In a further preferred form, the method produces an analogue of a heteromeric protein, wherein the target-site is a receptor, and the analogue is an agonist, partial agonist, antagonist, partial antagonist or reverse antagonist at the receptor.

In another form, the method produces an analogue of a heteromeric protein wherein one or more of the or each chemical cross-links comprise a covalent bond selected from the group including disulfide bonds, lactam bonds, tioesther bonds and thioether bonds.

In a further form, the method produces an analogue of a heteromeric protein wherein one or more of the or each chemical cross-links are introduced by a substitution of the first and/or second amino acids of the or each set.

In yet another form, the method produces an analogue wherein the substitution of the first and/or second amino acids of the or each set provides at least one disulfide bond as the chemical cross-link.

In yet a further form, the method produces an analogue of a heteromeric protein wherein one or more amino acids within the or each target site-binding subunits, other than the or each set of chemically cross-linked first and second amino acids, are optionally substituted to modify one or more biological activities of the analogue.

In a preferred form, the method provides an analogue of a heteromeric protein, the protein having a total subunit number of n equalling 2.

In yet another preferred form, the method produces an analogue of a relaxin superfamily protein, the analogue being an agonist, partial agonist, reverse agonist or antagonist at a relaxin superfamily receptor.

Preferably, the method produces an analogue of a B-chain of a relaxin superfamily member.

Even more preferably, the method produces an analogue of a B-chain of INSL3.

Preferably, the method produces an INSL-3 analogue which is constrained by a chemical cross-link between a first amino acid between positions 2 and 8 and a second amino acid between position 21 and 25 of the sequence set forth in SEQ ID NO:2.

In a preferred form, the first and second amino acids are substituted with cysteine residues and the chemical cross-link is a disulfide bond.

Preferably, the method produces an INSL3 analogue with a tryptophan residue in a C-terminal region.

In one preferred form, the method includes the synthesis of an INSL3 analogue selected from the sequences set forth in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.

In another form, the method produces an analogue of a B-chain of relaxin.

Preferably, the methods produces a relaxin analogue which is constrained by a chemical cross-link between a first amino acid between positions 2 and 8 and a second amino acid between position 21 and 25 of the sequence set forth in SEQ ID NO:1.

In another form, there is provided an analogue of a heteromeric protein synthesized according to the methods of the present invention.

In yet another form, there is provided a pharmaceutical composition including one or more of the analogues of the present invention, and pharmaceutically acceptable salts thereof.

In a further embodiment, the invention contemplates the use of the analogues and/or pharmaceutical compositions of the present invention in the treatment of conditions, the conditions including: hyperplastic disorders, neoplastic disorders, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Rayneud's disease, Rayneud's phenomenon, cryptorchidism, disregulation of spermatogenesis and reproductive development including descent of the gonads.

Sequence Listings

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<212> PRT
<213> peptide

<400> 1

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1 5 10 15

Arg Ala Gln Ile Ala Ile Cys Gly Met Ser Thr Trp Ser
20 25

<210> 2
<211> 31
<212> PRT
<213> peptide

<400> 2

Pro Thr Pro Glu Met Arg Glu Lys Leu Cys Gly His His Phe Val Arg
1 5 10 15

Ala Leu Val Arg Val Cys Gly Gly Pro Arg Trp Ser Thr Gln Ala
20 25 30

<210> 3
<211> 25
<212> PRT
<213> peptide

<400> 3

Ser Cys Met Glu Glu Val Ile Lys Leu Ser Gly Arg Glu Leu Val Arg
1 5 10 15

Ala Gln Ile Ala Ile Ser Gly Cys Ser

20 25

<210> 4
<211> 27
<212> PRT
<213> peptide

<400> 4

Thr Pro Cys Met Arg Glu Lys Leu Ser Gly His His Phe Val Arg Ala
1 5 10 15

Leu Val Arg Val Ser Gly Gly Pro Cys Trp Ser
20 25

<210> 5
<211> 27
<212> PRT
<213> peptide

<400> 5

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1 5 10 15

Leu Val Arg Val Ser Gly Gly Pro Cys Trp Ser
20 25

<210> 6
<211> 27
<212> PRT
<213> peptide

<400> 6

Thr Pro Cys Met Arg Glu Lys Leu Ser Gly Arg Glu Leu Val Arg Ala
1 5 10 15

Gln Val Ile Ala Ile Gly Gly Pro Cys Trp Ser
20 25

<210> 7
<211> 27
<212> PRT
<213> peptide

<400> 7

Thr Cys Glu Met Arg Glu Lys Leu Ser Gly His His Phe Val Arg Ala
1 5 10 15

Leu Val Arg Val Ser Gly Gly Cys Arg Trp Ser
20 25

Description of the Figures

FIGURE 1: Graph showing the binding of INSL3, cINSLa and cINSLb analogues to the LGR8 receptor.

FIGURE 2: Graph showing an absence of binding of the relaxin-like or INSL3 analogues to the LGR7 receptor.

FIGURE 3: Sequences and constraints of the native relaxin and INSL3-B chains and the exemplary analogues designed.

FIGURE 4: Graph showing the antagonistic activity of cINSLa in the inhibition of the response of INSL3 (measured by cAMP response) to the native INSL3 receptor (LGR8).

FIGURE 5: CD spectra of cINSLa showing significant alpha-helical content in water and phosphate buffered saline.

Detailed Description

In its broadest form, the present invention provides in one aspect the method of synthesing an analogue of a heteromeric protein, with a binding activity at a target site. In a preferred aspect, the invention provides an analogues which is an agonist, partial agonist, antagonist, partial antagonist or reverse agonist, at the receptor of a relaxin superfamily member.

In one of the surprising findings of this invention, applicants have found that a relaxin superfamily analogue, which is an agonist or antagonist to a relaxin superfamily receptor, may be provided by a relaxin superfamily B chain which is constrained by chemical cross linking, most preferably between a residue between positions 2 and 8, with a residue between positions 21 and 25 of the respective relaxin superfamily B chain sequences.

In its broadest form, the present invention provides a method of producing an analogue of a heteromeric protein with a binding activity at a target site, the heteromeric protein having a total subunit number of n, the method including:

- synthesizing one or more target-site binding subunits of the heteromeric protein, wherein the maximum number of active subunits identified is n-1; and
- identifying at least one set of a first and a second amino acid on respective, opposing strands of a turn and/or loop moiety in the or each active subunits of the protein, with an alpha-alpha or beta-beta carbon separation distance of less than six angstroms; and
- introducing a chemical cross-link between the first and second amino acids of the at least one set of first and second amino acids;

wherein the chemical cross-link stabilises a three dimensional structure of at least one subunit.

In a further preferred form, the method produces an analogue of a heteromeric protein, wherein the target-site is a receptor, and the analogue is an agonist, partial agonist, antagonist, partial antagonist or reverse antagonist at the receptor.

In another form, the method produces an analogue of a heteromeric protein wherein one or more of the or each chemical cross-links comprise a covalent bond selected from the group including disulfide bonds, lactam bonds, tioesther bonds and thioether bonds.

In a further form, the method produces an analogue of a heteromeric protein wherein one or more of the or each chemical cross-links are introduced by a substitution of the first and/or second amino acids of the or each set.

In yet another form, the method produces an analogue wherein the substitution of the first and/or second amino acids of the or each set provides at least one disulfide bond as the chemical cross-link.

In yet a further form, the method produces an analogue of a heteromeric protein wherein one or more amino acids within the or each target site-binding subunits, other than the or each set of chemically cross-linked first and second amino acids, are optionally substituted to modify one or more biological activities of the analogue.

In a preferred form, the method provides an analogue of a heteromeric protein, the protein having a total subunit number of n equalling 2.

In yet another preferred form, the method produces an analogue of a relaxin superfamily protein, the analogue being an agonist, partial agonist, reverse agonist or antagonist at a relaxin superfamily receptor.

Preferably, the method produces an analogue of a B-chain of a relaxin superfamily member.

Even more preferably, the method produces an analogue of a B-chain of INSL3.

Preferably, the method produces an INSL-3 analogue which is constrained by a chemical cross-link between a first amino acid between positions 2 and 8 and a second amino acid between position 21 and 25 of the sequence set forth in SEQ ID NO:2.

In a preferred form, the first and second amino acids are substituted with cysteine residues and the chemical cross-link is a disulfide bond.

Preferably, the method produces an INSL3 analogue with a tryptophan residue in a C-terminal region.

In one preferred form, the method includes the synthesis of an INSL3 analogue selected from the sequences set forth in SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.

In another form, the method produces an analogue of a B-chain of relaxin.

Preferably, the methods produces a relaxin analogue which is constrained by a chemical cross-link between a first amino acid between positions 2 and 8 and a second amino acid between position 21 and 25 of the sequence set forth in SEQ ID NO:1.

In another form, there is provided an analogue of a heteromeric protein synthesized according to the methods of the present invention.

In yet another form, there is provided a pharmaceutical composition including one or more of the analogues of the present invention, and pharmaceutically acceptable salts thereof.

In a further embodiment, the invention contemplates the use of the analogues and/or pharmaceutical compositions of the present invention in the treatment of conditions, the conditions including: hyperplastic disorders, neoplastic disorders, neurological disorders, angiogenic disorders, cardiovascular disorders, female reproductive disorders, conditions associated with pregnancy, renal disease, inflammatory bowel disease, Rayneud's disease, Rayneud's phenomenon, cryptorchidism, disregulation of spermatogenesis and reproductive development including descent of the gonads.

The chemical cross-link may be a disulfide bond between cysteine residues introduced into the relaxin superfamily member B chain sequence at a position between residues 2 and 8, and a second cysteine residue introduced between positions 21 and 25. Other chemical cross-links may be used. Examples include lactam bonds, ethers, thioethers, thioesters. By way of example, chemical cross-links may be used as described in WO 00/75176 and WO 01/52875, both of which are incorporated herein by reference.

Amino acids of the relaxin superfamily B chain sequences may be substituted by conservative amino acid substitutions, for example when an amino acid is replaced by one of similar size and with similar charge properties. Examples of amino acid substitutions include those described in WO 01/52875, which as mentioned above is incorporated herein by reference.

Amino acids of the relaxin superfamily members B chains may be truncated. For example one or more amino acids may be truncated from the N and/or C terminus, for example from one to five amino acids from one or both N and/or C termini.

Analogues may include amino acids that improve solubility, for example *in vivo* half life, and/or retention of α-helix structure within the peptide sequences. Such residues include stearically hindered and helix promoting amino acids such as α-aminoisobutryic acid (Aib).

This invention will now be described with reference to the following examples.

Example 1

Methods and Materials

Molecular design of INSL3 analogues

Using SYBYL molecular modelling software (Tripos) on a Silicon Graphics O2 workstation, we designed a model of INSL3 B-chain from the X-ray crystal structure of human Gene 2 relaxin B-chain as a template. After modifying the sequence to resemble that of INSL3, an energy minimisation using a Tripos forcefield with Gasteiger-Marsili charges was carried out. From the resulting energy minimised model, two residues were identified (Glu^4 and Arg^{26}) to have Cβ atoms within 4Å of each other. These residues were then replaced with cysteine and a disulphide bond formed to give the cyclic peptide cINSLa. A second cyclic analogue cINSLb by changing His^{12} to Arg, to mimic a relaxin-like binding motif of Arg-X-X-Arg-X-X-X-Val.

Solid-phase peptide synthesis:

The synthesis of the INSL3 analogues were achieved using the continuous flow Fmoc-methodology as previously described (Dawson, 1999). The solid support was Fmoc-PAL PEG-PS (PerSeptive Biosystems, USA), and HBTU-activated Fmoc-amino acids were used throughout.. N^{α}-Fmoc deprotection was with 20% piperidine in DMF. All derivatives

were purchased from Auspep (Melbourne, Australia). Cleavage of the peptides from the solid support and side chain deprotection was achieved by a 3 hour treatment with trifluoroacetic acid (TFA) in the presence of phenol, thioanisole, ethanedithiol and water (82.5/5/5/2.5/5, v/v). The crude peptides were subjected to reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac C18 column (Hesperia, USA) using a 1%/min gradient of CH3CN in 0.1% aqueous TFA. Peptides were then oxidised in a buffer containing 10% dimethylsulfoxide (DMSO) for 24 hours and the reaction monitored on HPLC and by mass spectrometry.

Characterization:

The purity of the synthetic peptide was assessed by analytical RP-HPLC, and matrix-assisted laser desorption time of flight (MALDITOF) mass spectrometry using a Bruker Biflex instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptide quantitation was by amino acid analysis of a 24 hour acid hydrolyzate using a GBC instrument (Melbourne, Australia).

Circular dichroism (CD) spectroscopy:

CD spectra were taken on a Jasco J-720 instrument at room temperature in a 1 mm path-length cell, Doubly distilled water, 10 mM sodium phosphate buffer containing 120 mM NaCl pH 7.4, and spectroscopy grade trifluoroethanol (TFE) were used as solvents. The peptide concentrations were made to 1μM, determined by quantitative RP-HPLC and amino acid analysis (ref). Curves were smoothed by the algorithm provided by Jasco, Mean residue ellipticity ($[q]_{MR}$) is expressed in degrees^{x}cm2/dmole by using the molecular mass. CD spectra evaluations were based on comparison with known peptide conformations (Horvat, 1999).

Functional cAMP assays;

Human 293T cells derived from human embryonic kidney fibroblasts were maintained and

transfected with LGR7, or LGRB expression plasmids as previously described [ref]. Cells were preincubated in the presence of 0.25mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma) before various treatments for 30 minutes. At the end of the incubation cells were lysed for measurement of cAMP using a well characterized cAMP ELISA [ref]. All experiments were repeated at least three times using cells from independent transfections, Results are plotted as pmol cAMP/10^4 cells.

Ligand binding assays:

The method for binding of 33P-labelled relaxin to 293T cells stably transfected with either LGR7 or LGR8 has been described previously [ref]. Data are expressed as mean ± SEM of % specific binding of triplicate determinations performed on a least three independent plates of transfected cells. Data were plotted using the one-site competition functions of the PRISM program (Graphpad Inc., San Diego, USA).

Example 2

Sites for the incorporation cyclising constraints to create B chain analogues were determined by molecular modelling studies. In the case of relaxin, distances between β-carbon atoms in the B chain were measured in the X-ray crystal structure of relaxin [6RLX], using the molecular modelling program Sybyl (Tripos Inc St Louis MO). Residue 25 (Met) within the C chain helix and residue 3 (Trp) in the strand were found to have a β-carbon to β-carbon distance of 3.855 angstroms. These residues were thus deemed suitable for replacement by Cys and subsequent formation of a disulphide bond. This constrained two ends of the molecule in a similar fashion to the native protein, resulting in the cyclic relaxin B chain mimetic 3. Methods are as described in International Patent Applications Nos. WO 00/75176 and WO 01/52875.

In the case of INSL3 we constructed a model of the 3D structure of INSL3 by substituting the appropriate amino acids into the relaxin structure, and carrying out an energy minimisation of the resultant INSL3 B chain. We then identified pairs of residues as potential sites for the incorporation of a Cys to Cys constraint, including the pair Glu4 and

Arg26, residues adjacent to those replaced in the relaxin analogue, giving the cyclic INSL3 mimetic 4 (Figure 3).

Following the biological characterisation of peptides 3 and 4 (Figure 3) additional compounds were prepared incorporating the Cys to Cys constraint of peptide 4:

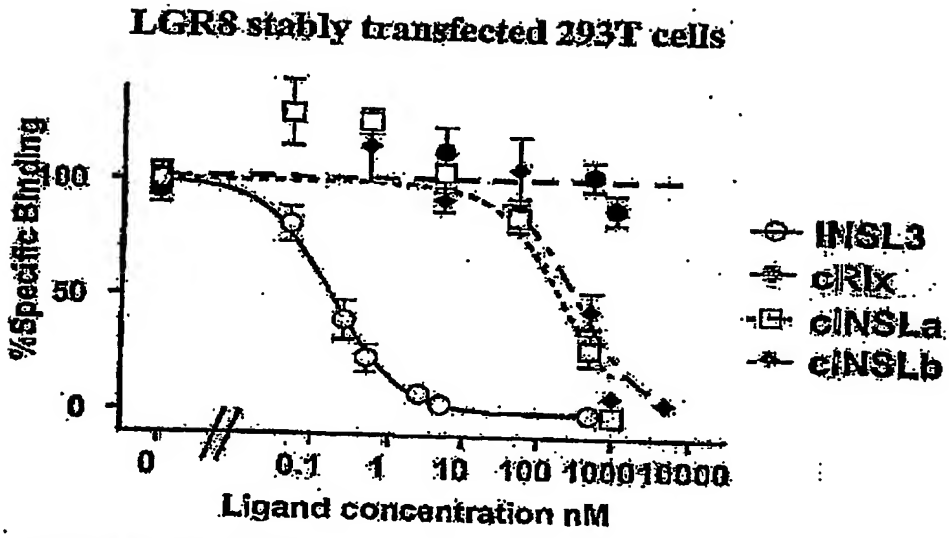
- peptide 5, a INSL3-based sequence, in which His12 form the INSL3 sequence was replaced by Arg in an attempt to obtain an analogue of the putative relaxin receptor binding cassette which if absent in INSL3;
- peptide 6, a hybrid sequence in which the helix of relaxin B chain (residues 13 to 22) was substituted into the INSL3 peptide 4, for similar reasons described fore peptide 5.

We also prepared peptide 7, an INSL3 mimetic with a Cys to Cys constraint in position 3 and 25.

The same approach is used to preapre insulin B chain analogues and other relaxin superfamily analogues.

Although several preferred embodiments have been described in detail, it should be understood that various changes, substitutions, and alterations can be made herein by one ordinarily skilled in the art without departing from the spirit or scope of the present invention.

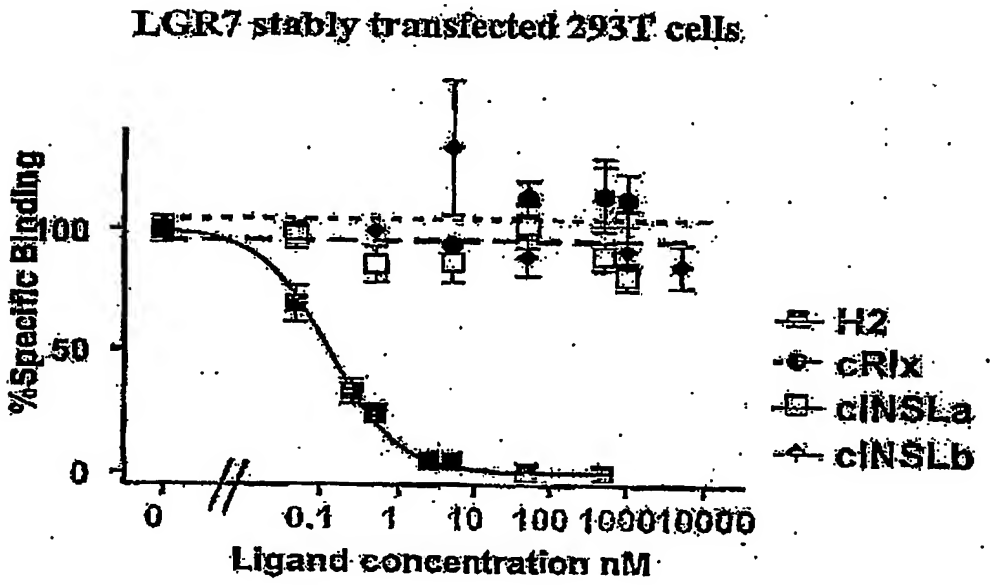
Figure 1



LGR8 -pEC_{50} (n=3/4)

INSL3 - 9.72 ± 0.16
GB -0
GB II - 6.79 ± 0.27
GB III - 6.26 ± 0.23
} NS

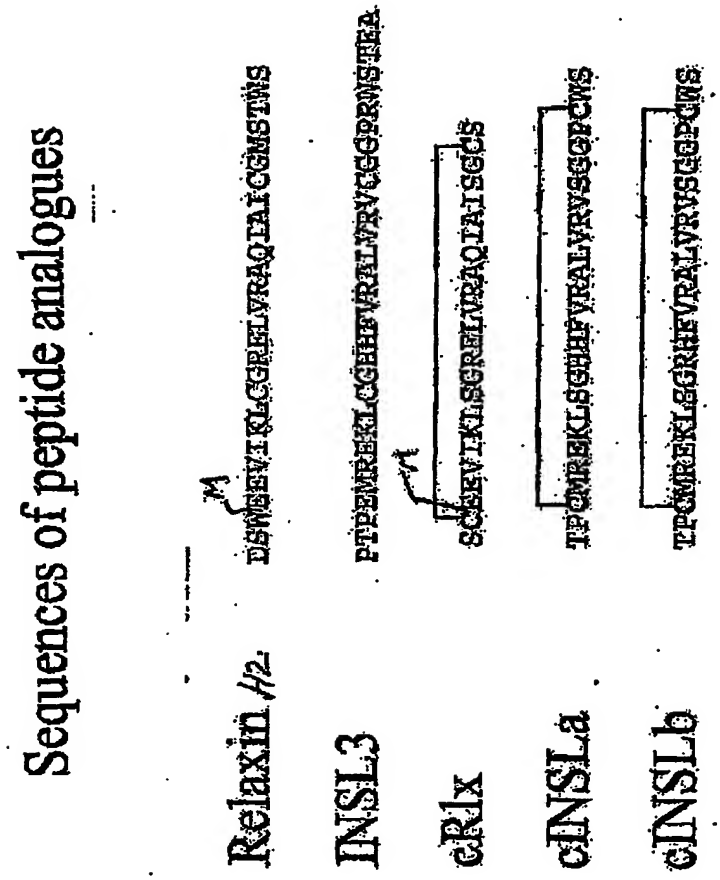
Figure 2



LGR7 -pEC_{50} (n=3/4)

H2 - 10.04 ± 0.37
cRlx - 0
cINSLa II - 0
cINSLb - 0

Figure 3


Sequences of peptide analogues
Relaxin H2
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INSL3
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cR1x
SCMEVIKLSCRELVRAQIAISGDS
cINSLa
TPGMREKLSCHEIRTALVRVSGPCWS
cINSLb
TPGMREKLGCRHEVRALVRVSGGPCWS

Figure 4

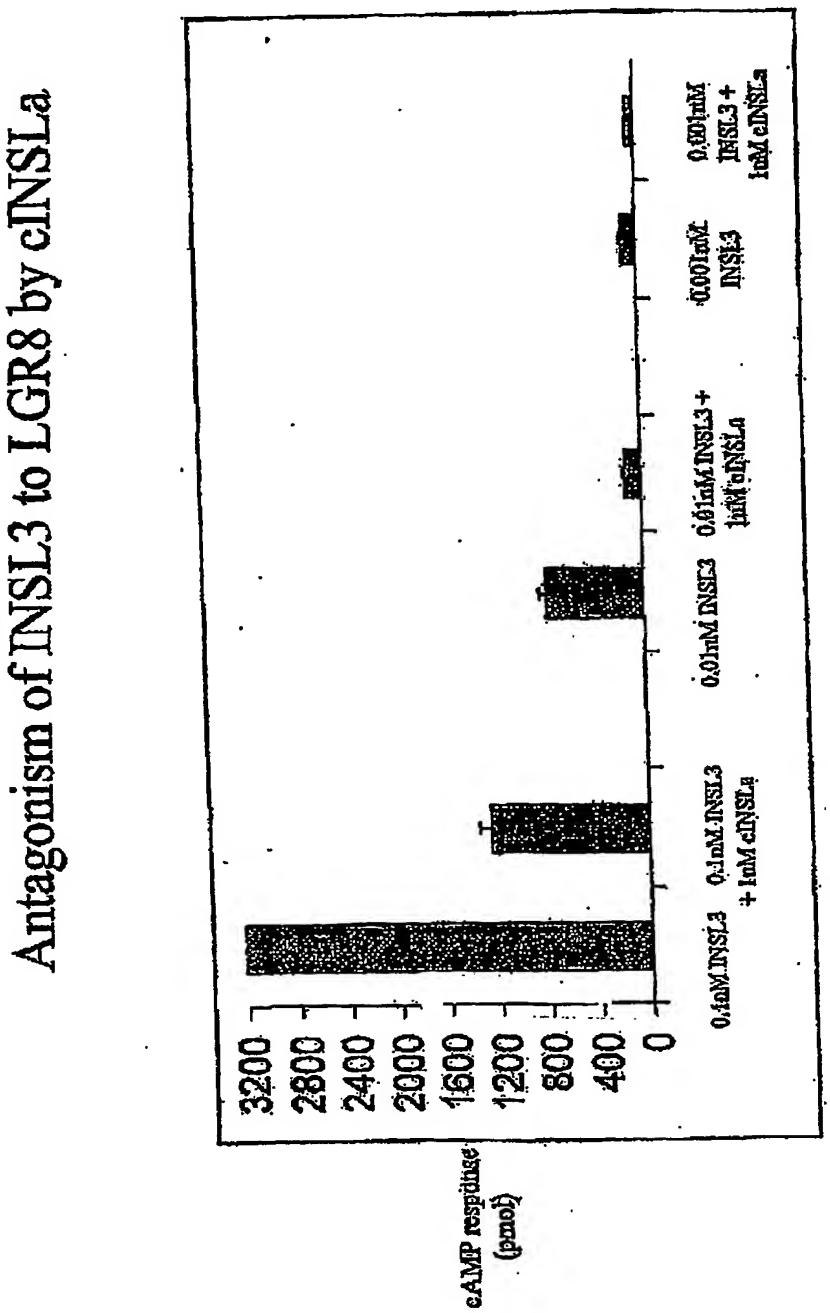
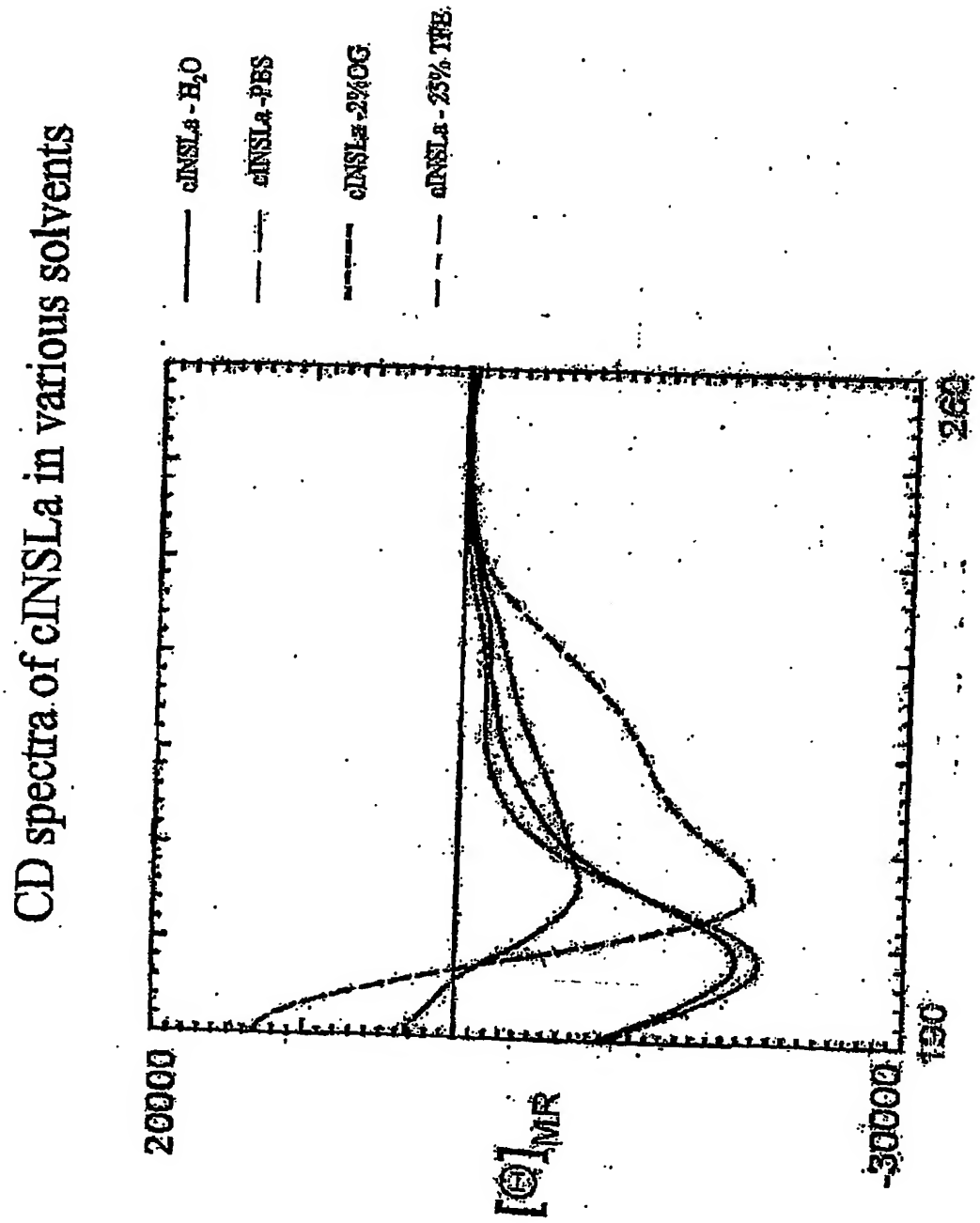

Antagonism of INSL3 to LGR8 by cINSLa
cAMP response (pmol)
3200
2800
2400
2000
1600
1200
800
400
0
0.1nM INSL3
0.1nM INSL3 + 1nM cINSLa
0.01nM INSL3
0.01nM INSL3 + 1nM cINSLa
0.001nM INSL3
0.001nM INSL3 + 1nM cINSLa

Figure 5


CD spectra of cINSLa in various solvents
cINSLa - H_2O
cINSLa -PBS
cINSLa -2%OG
cINSLa - 25% TFE
20000
-30000
260
190
$[\Theta]_{MR}$

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